Targeting FPR1 and CXCR4 in cancer and the contribution of the tumor microenvironment

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Chapter 1

General introduction

Glioblastoma

Glioblastoma (GBM) is the most common type of primary brain tumors with an average incidence of approximately 3.1 per 100,000 adults per year [1,2]. Current treatment options, consisting of surgical resection followed by radiotherapy and temozolomide, result in a 14 months median survival in newly diagnosed GBM patients [3,4].

GBM features include rapid and invasive tumor growth, hypoxia and vascularization. This generates a network of disorganized, tortuous and permeable vessels. A key element of angiogenesis is the vascular endothelial growth factor-A (VEGF-A), which is highly expressed in brain tumors. Rapid tumor growth and oxygen depletion induce the upregulation of hypoxia inducible factor 1-alpha (HIF1α), of VEGF-A and a subsequent cascade of events. In this cascade bone marrow derived cells (BMDC) are recruited to the tumor site, alternative vascularization pathways are activated and a vast number of growth factors are upregulated. GBM is characterized by tumor cell migration into the surrounding brain parenchyma [5]. Therefore complete eradication of these tumors by local surgical resection and radio-chemotherapy remains almost unachievable.

The G-Protein Coupled Receptor (GPCR) family in cancer

Amongst all the membrane bound receptors, the superfamily of G-protein coupled 7 transmembrane cell surface receptors are of remarkable diversity, high promiscuity and variable specificity for their ligands. With regard to this, chemokine receptors and formyl peptide receptors are characterized by very high affinity for their ligands. GPCRs are chemoattractant receptors and transducers of extracellular signals to intracellular effector pathways. Signal transduction occurs by activating heterotrimeric G-proteins, constituted by the α, β and γ subunits. Ligand binding results in the release of an α -subunit with subsequent inhibition of adenyl cyclase and release of the βγ-complex, which activates the MAP kinase pathways and phospholipase C (PLC). This

ultimately induces calcium release from the endoplasmic reticulum [9]. GPCR upregulation is observed in various tumor types and is therefore explored as target for anti-cancer therapy [10]. Aberrant GPCR functions are implicated in many facets of cancer growth, including cancer cell proliferation, metastasis and tumor induced angiogenesis, progression of hormone refractory cancers and endocrine tumors [11]. In GBM, GPCRs play a role in tumor growth, invasion and production of angiogenic factors by sensing cognate ligands produced by the microenvironment [11-14].

Chemoattractant receptors of the GPCR superfamily include FPR1 and CXCR4. FPR1 was originally identified as a mediator of leukocyte migration and was characterized by its binding to N-formyl peptides of bacterial and mitochondrial origins. In humans the only conserved N-formyl peptides originate from cleavage products of mitochondrial proteins. Specifically the release of mitochondrial peptides from necrotic tumor tissue constitutes a source of ligands for FPR1. Chemotaxis inhibitory protein of S. aureus (CHIPS) is an anti-inflammatory compound secreted by S. aureus and very potent inhibitor of FPR1. Recently the expression of FPR1 was identified on tumor cells where its activation induces a number of downstream signaling events linked to tumor angiogenesis and migration.

Various studies have reported that CXCR4 is over-expressed by cancer cells, bone marrow derived and stromal cells. In prostate cancer cells and in vivo models, CXCR4 plays an important role in tumor growth, angiogenesis and the interaction of malignant cells with their microenvironment [15,16]. AMD3100 is a potent CXCR4 inhibitor, which blocks the binding pocket of CXCR4. In vivo studies showed that AMD3100 treatment reduces metastasis and sensitizes tumor cells to anti-cancer drugs [16-18].

Scope and Aim of this thesis

The aim of this thesis was to investigate the interaction of GBM and prostate cancer cells with their tumor microenvironment and the role of the GPCRs

FPR1 and CXCR4 in this process.

In Chapter 2 we reviewed the literature for molecular mechanisms of neovascularization following resistance to the VEGF-A antibody bevacizumab in GBM patients, with special emphasis on the recruitment of BMDCs. In addition we evaluated which key factors are consistently upregulated following bevacizumab therapy. Based on that, we proposed a rationale for possible therapeutic combination options with bevacizumab.

The role of tumor-associated macrophages (TAMs) as vascular modulators was further investigated in Chapter 3. In this chapter we aimed at exploring the expression of Ang1, Ang2 and Tie-2 in a series of human GBM samples and determined the extent to which a subclass of TAMs called Tie-2 expressing monocytes (TEM), was present in GBM. Additionally we correlated this data with patient characteristics and data including tumor microvessel density, proliferation fraction, apoptotic fraction and VEGF-A protein expression.

In Chapter 4 we studied the effect of CXCR4 inhibition combined with irradiation in prostate cancer cells. In vitro, we investigated how the human prostate cancer cell lines PC3-Luc and LNCaP, when co-cultured with stromal cells and treated with the CXCR4 inhibitor AMD3100, were sensitized to irradiation. In mice xenografted with the luciferase-expressing PC3-Luc cells, tumor growth and metastasis were evaluated after daily treatment with AMD3100 or irradiation either alone or combined. The amount of circulating tumor cells (CTCs) was evaluated by bioluminescent imaging of blood samples collected before and at short time intervals after AMD3100 injections.

Next we studied the role of FPR1 in GBM. The activation of FPR1 on the human astrocytoma cell line U87 promotes in vitro cell motility, growth and angiogenesis. In Chapter 5 we investigated the capacity of the bacterial ligand fMLF to activate FPR1 on U87 cells and explored the FPR1 inhibitor CHIPS, as a potential anti-glioma drug. Calcium mobilization and migration assays served as read outs for the activation of FPR1 with respectively mitochondrial and bacterial ligands on transfected U937FPR and U87 cells. Additionally U87

xenografts in NOD-SCID mice served to investigate the effects of CHIPS in vivo. We further investigated the presence of FPR1 in GBM, which is typically characterized by necrosis and might contain mitochondrial peptides released from ruptured cells. Therefore In Chapter 6 we evaluated the expression of FPR1 in human GBM tumor tissue with immunohistochemistry. In addition in early passages of human GBM cell lines we assessed the functional presence of FPR1 with fMLF induced calcium mobilization assays. In U87 we further analyzed the effect of FPR1 activation with mitochondrial peptides using calcium mobilization, FPR1 downstream protein phosphorylation and migration assays. We also tested whether CHIPS could inhibit these responses. To confirm the presence of FPR1 on tumor cells in GBM tissue, we applied immunofluorescence double staining of FPR1 with the glial marker GFAP and with the macrophage markers CD68/CD163 on GBM patient samples. Finally ex vivo, FPR1 expression in orthotopic brain tumor xenografts of the primary GBM cell lines was determined with immunohistochemistry.

The pharmokinetics and toxicity of CHIPS have been previously tested in a small clinical phase 1 study in which CHIPS was administered as an anti-inflammatory agent. This study revealed that upon intravenous administration of CHIPS, the pre-existent anti-CHIPS antibodies in circulation caused toxicities. This generates the necessity to search for a CHIPS variant with less immunogenic properties.

The aim of the research performed in the next chapter was to design an optimal new CHIPS variant that allows the use of a concentration sufficient to effectively inhibit FPR1 while inducing less host antibody driven toxicity. In Chapter 7 we therefore used an Escherichia Coli expression system to develop less immunogenic CHIPS mutants. Cloning procedures served to investigate the binding properties of mutant CHIPS proteins obtained by altering elements in the protein structure. The inhibitory effects of CHIPS on FPR1 was tested with calcium mobilization assays. Furthermore truncated parts of the N-terminus of CHIPS were tested in migration assays and truncated

parts of the N-terminus of the FPR1-like binding protein (FLIPrL; a FPR1 high affinity CHIPS homologue) were tested with calcium mobilization and migration assays. Additionally we cloned a less immunogenic CHIPS protein variant (CHIPS-JC) and performed a direct ELISA to test its affinity with human serum anti-CHIPS IgG. Calcium mobilization and migration assays were used to test the inhibitory effects of CHIPS-JC on FPR1. Finally in Chapter 8 the experimental results of this thesis are summarized, followed by a discussion and possible directions for further research.

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